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(54) Title: MODIFIED PROTEIN MATRICES

(57) Abstract: Matrices formed of materials which bind to growth factors, directly or indirectly, are used for controlled delivery of the growth factors, especially for use in tissue repair and/or regeneration. The matrices may have heparin bound thereto ionically or covalently, or heparin-like binding sites incorporated in the matrix forming materials. The heparin or heparin-like binding sites can bind to the growth factors. The matrices can be implanted directly, alone or in combination with cells, or implanted and seeded with cells, at a site where tissue repair or regeneration is desired. A particularly preferred application is in regeneration and repair of nerves. The matrices can be coated on medical devices and implants. The matrices most preferably encourage and promote cellular ingrowth.

MODIFIED PROTEIN MATRICES

Background of the Invention

The present invention relates to the field of three-dimensional matrices that contain pharmacologically active molecules, particularly growth factors, most especially growth factors with low heparin-binding affinity, and the use thereof to release the growth factors and to promote cell and tissue growth.

Many growth factors are thought of as "heparin-binding" growth factors. Families with one or more members that bind heparin include fibroblast growth factors and bone morphogenetic proteins (BMPs) (Presta, et al., (1992) *Biochem Biophys Res Commun* 185: 1098-1107; Reddi, A. (1998) *Nature Biotechnology* 16: 247-252; McCaffrey, et al. (1992) *J Cell Physiol* 152: 430-440)). Additional growth factors that bind heparin include transforming growth factor beta 1 (TGF-beta 1), interleukin-8, neurotrophin-6, vascular endothelial cell growth factor, heparin-binding epidermal growth factor, hepatocyte growth factor, connective tissue growth factor, midkine, and heparin-binding growth associate molecule (Spillmann, et al., (1994) *Nature* 372: 266-269; Tessler, et al., (1994). *J Biol Chem* 269: 12456-12461; Kiguchi, et al., (1998) *Mol Carcinog* 22: 73-83; Kinosaki, et al., (1998) *Biochim Biophys Acta* 1384: 93-102; Steffen, et al., (1998) *Growth Factors* 15: 199-213; Kaneda, et al., (1996) *J Biochem (Tokyo)* 119: 1150-1156; Nolo, et al. (1996). *Eur J Neurosci* 8: 1658-1665)). These factors have shown the potential to enhance healing in many different types of tissue including vasculature, skin, nerve, and liver.

Controlled delivery devices based on heparin-affinity of these growth factors have been designed previously (Edelman, et al., (1991) *Biomaterials* 12: 612-626; DeBlois, et al., (1994) B*iomaterials* 15: 665-672; Downs, et al., (1992) *J Cell Physiol* 152: 422-429). These drug delivery devices have previously been used to deliver "heparin-binding" growth factors. Such

"heparin-binding" growth factors are typically considered to be those which bind to heparin with a relatively high affinity, often characterized by elution from heparin-affinity columns at NaCl concentrations well above physiological levels (greater than 140 mM). In such delivery systems, the heparin-binding affinity of the growth factor is usually used to sequester the growth factors to immobilized heparin of some form. For example, Edelman et al. used heparin-conjugated SEPHAROSE™ beads to bind basic fibroblast growth factor (bFGF) and then encapsulated the beads with alginate (Edelman, et al., (1991); Edelman, et al., (1992) *Controlled Release Systems Containing Heparin and Growth Factors*, MIT: USA). These beads serve as reservoirs that release bFGF slowly based on the binding and dissociation constants of bFGF and heparin.

The delivery of "non-heparin-binding growth factors" has previously required release methods for delivery typically based on diffusion-controlled release of the factors from porous materials (Houle and Johnson (1989) *Neurosci Lett* 103: 17-23; Camarata, et al. (1992) *Neurosurgery* 30: 313-319; Powell, et al., (1990) *Brain Res* 515: 309-311; Maysinger, et al., (1992) *Neurosci Lett* 140: 71-74). There remains a need in the medical arts for a device that is capable of providing the release of low heparin-binding growth factors at a controlled and predictable rate in order to provide effective release of the factor over a clinically useful period during the wound healing process.

It is therefore an object of the present invention to provide matrices that provide for the controlled release of growth factors, which are useful in tissue regeneration or repair.

Summary of the Invention

Matrices formed of materials which bind to growth factors, directly or indirectly, are used for controlled delivery of the growth factors, especially for use in tissue repair and/or regeneration. The matrices may have heparin bound thereto ionically or covalently, or heparin-like binding sites incorporated in the matrix forming materials. The heparin or heparin-like binding sites can bind to the growth factors. The ratio of binding sites to

peptides or growth factors can be used to vary the kinetics of the binding, thereby providing a means to increase or decrease the rate of association of the growth factors with the matrix and thus the rate of delivery. A ratio of at least 1:1 heparin to growth factor is preferably used. Increasing the ratio decreases the rate of release. The dissociation kinetics of low affinity heparin-binding proteins are relatively fast, but a high number of binding sites allows rebinding of the growth factor before it can diffuse out of the matrix. Release can occur by diffusion of the growth factor out of the matrix prior to rebinding, or it can occur if the growth factor encounters a cell surface receptor before rebinding to a heparin site. The most preferred matrix is heparin or peptides containing heparin binding sites bound to fibrin, capable of attaching heparin, a heparin-like polysaccharide, or a heparin-like polymer, and a growth factor or peptide fragment thereof having a basic domain that binds heparin.

In one embodiment, the preferred matrix is fibrin bound to heparin or peptides containing heparin-like binding sites, capable of attaching heparin, a heparin-like polysaccharide, or a heparin-like polymer, and a growth factor or peptide fragment thereof having a basic domain that binds heparin with low affinity, i.e., a peptide/protein that will elute from a heparin affinity column at an NaCl concentration of between about 25 mM to about 140 mM. Preferred "low heparin-binding affinity" growth factor or peptide fragments thereof have a length of about 8 to 30 amino acid residues. Examples of low heparin-binding affinity growth factor include neurturin, persephin, IGF-1A, IGF-1, EGF, NGF, NT-3, BDNF, NT-4, TGF-beta2, TGF-beta3, TGF-beta4, or a peptide fragment of any one of these.

The matrices can be modified to include chemically cross-linked peptides. These peptides preferably include a sequence that mimics the heparin-binding regions of a protein such as neural cell adhesion molecule, fibronectin, laminen, midkine, and antithrombin III. These peptides are chemically cross-linked to the matrix to enhance attachment of cells to the matrix. Representative peptides include HAV (SEQ. I.D. NO. 6) bound to the matrix at a concentration of about 2 mol peptide/mol fibrin gel; RGD

(SEQ. I.D. NO. 2) bound to the matrix at a concentration of about 1.5 mol RGD/peptide mol fibrin gel; IKVAV (SEQ. I.D. NO. 1) bound in a concentration of about 8 mol peptide/mol fibrin gel; YIGSR (SEQ. I.D. NO. 3) at a concentration of about 6 mol peptide/mol fibrin gel and RNIAEIIKDI (SEQ. I.D. NO. 5) at a concentration of about 8 mol peptide/mol fibrin gel, or mixtures thereof of two or more peptides or fusion peptides including these or similar peptides. In one preferred embodiment for neurite extension, HAV (SEQ. I.D. NO. 6) and RNIAEIIKDI (SEQ. I.D. NO. 5) are included at a ratio of about 1:3. In another embodiment RGD (SEQ. I.D. NO. 2) and YIGSR (SEQ. I.D. NO. 3) or DGEA (SEQ. I.D. NO. 4) are combined with a ratio of about 1:3. In still another example, mixtures of peptides include RGD (SEQ. I.D. NO. 2), YIGSR (SEQ. I.D. NO. 3), and RNIAEIIKDI (SEQ. I.D. NO. 5) at a ratio of about 1:1:1, respectively.

In still another embodiment, the peptide includes two or more different domains, at least one of which is a heparin-binding domain and at least one additional domain is a bioactive molecule or binds to another molecule, i.e., includes a second binding domain. Examples of other binding domains K(A)FAKLAARLYRKA (SEQ. I.D. NO. 8), YKKIIKKL (SEQ. I.D. NO. 9), KHKGRDVILKKDVR (SEQ. I.D. NO. 10), or a mixture thereof. An exemplary heparin binding domain from ATIII is SEQ. I.D. NO.18. In one embodiment, the preferred heparin-binding domain elutes from a heparin-affinity column at [NaCl] greater than 0.34 mol. Others include alpha 2 protease inhibitor and a modified aprotinin with a Factor XIIIa substrate site. These peptides may also include an enzymatic cleavage site, most preferably having a low kcat and a high km. An Exemplary chimeric peptide includes a first C-terminal domain comprising a heparin-binding peptide sequence of antithrombin III; and a second N-terminal domain comprising an alpha 2-plasmin inhibitor substrate for Factor XIIIa.

The matrices can be implanted directly, alone or in combination with cells,or implanted and seeded with cells, at a site where tissue repair or regeneration is desired. A particularly preferred application is in regeneration and repair of nerves. The matrices can be coated on medical

devices and implants. The matrices most preferably encourage and promote cellular ingrowth.

Brief Description of the Drawings

Figure 1 is a graph of the effect of matrix bound basic fibroblast growth factor on neurite extension from dorsal root ganglia in three-dimensional fibrin gels. Ganglia were dissected from day 8 chick embryos and placed in prewashed fibrin gels. * denotes $p<0.05$ versus unmodified fibrin. The soluble treatments contain bFGF, but not heparin or heparin-binding peptide. These treatments show no difference from unmodified fibrin suggesting that the washing protocol used was sufficient to remove unbound growth factor and that peptide, heparin, and growth factor is all necessary components of the controlled release system.

Bar 1, fibrin; Bar 2, with Peptide + heparin; Bar 3, 0.1, μg/ml bound; Bar 4, 1.0 μg/ml bound; Bar 5, 1.0 μg/ml soluble; Bar 6, 5.0 μg/ml bound; Bar 7, 5.0 μg/ml bound; Bar 8, 1.0 μg/ml culture; Bar 9, VEGF 1.0 μg/ml; Bar 10, 1.0 μg/ml bound to 50% peptide; Bar 11, 1 μg/ml + soluble ATIII; Bar 12, 1 μg/ml + heparin (no peptide). "Bound" refers to fibrin, with Peptide and heparin. "Soluble" refers to fibrin.

Figure 2 is a graph showing the effect of matrix bound NGF-beta, NT-3 and BDNF on neurite extension from dorsal root ganglia in three-dimensional fibrin gels. Ganglia were dissected from day 8 chick embryos and placed in prewashed fibrin gels. * denotes $p<0.05$ versus unmodified fibrin. The soluble treatments contain growth factor, but not heparin or heparin-binding peptide. These treatments show no difference from unmodified fibrin suggesting that the washing protocol used was sufficient to remove unbound growth factor.

Bar 1, fibrin; Bar 2, NGF bound at 0.1μg/ml; Bar 3, NGF soluble at 0.1μg/ml; Bar 4 BDNF bound at 0.1μg/ml; Bar 5, BDNF soluble at 0.1, μg/ml bound; Bar 6, NT-3 bound at 1.0 μg/ml; Bar 7, NT-3 soluble at 0.1 μg/ml bound. "Bound" refers to fibrin, with Peptide and heparin. "Soluble" refers to fibrin.

Figure 3 is a graph showing the ability of matrix bound NGF-beta to promote neurite extension from dorsal root ganglia in three-dimensional fibrin gels as a function of wash time prior to cell seeding. Ganglia were dissected from day 8 chick embryos and placed in prewashed fibrin gels. * denotes $p<0.05$ versus unmodified fibrin. The soluble treatments contain growth factor, but not heparin or heparin-binding peptide. This assay demonstrates that the matrix bound NGF- maintains its activity for 4 days while the unbound NGF- is not active after 1 day of washing. --■-- fibrin; --◆-- ngf bound (100 ng/ml); --●-- ngf soluble (100 ng/ml)

Figure 4 is a graph showing enhancement of neurite outgrowth with HAV (SEQ. I.D. NO. 6) modified fibrin gels. A concentration series of HAV (SEQ. I.D. NO. 6) cross-linked into fibrin was tested in triplicate with day 8 chick dorsal root ganglia. The level of growth at 24 and 48 hours was calculated and normalized to growth in unmodified fibrin. Mean and standard error of the mean are shown. Legend to Figure 1: -- 2 4 Hours, -◊- 48 Hours.

Figure 5 is a graph showing enhancement of neurite outgrowth with RGD (SEQ. I.D. NO. 2)-modified fibrin gels. A concentration series of RGD (SEQ. I.D. NO. 2) cross-linked into fibrin was tested in triplicate with day 8 chick dorsal root ganglia. (because fibrin naturally contains two active RGD (SEQ. I.D. NO. 2) sites, the level of incorporation of RGD (SEQ. I.D. NO. 2) becomes 2-10 mol RGD (SEQ. I.D. NO. 2)/mol fg instead of 0-8) The level of growth at 24 and 48 hours was calculated and normalized to growth in unmodified fibrin. Mean and standard error of the mean are shown. Legend to Figure 2: -- 2 4 Hour Data, -◊- 48 Hour Data.

Figure 6 is a graph showing enhancement of neurite outgrowth with IKVAV (SEQ. I.D. NO. 1)-modified fibrin gels. A concentration series of IKVAV (SEQ. I.D. NO. 1) cross-linked into fibrin was tested in triplicate with day 8 chick dorsal root ganglia. The level of growth at 24 and 48 hours was calculated and normalized to growth in unmodified fibrin. Mean and standard error of the mean are shown. Legend to Figure 3: -- 2 4 Hours, -◊- 48 Hours

Figure 7 is a graph showing enhancement of neurite outgrowth with RNIAEIIKDI (SEQ. I.D. NO. 5) modified fibrin gels. A concentration series of RNIAEIIKDI (SEQ. I.D. NO. 5) cross-linked into fibrin was tested in triplicate with day 8 chick dorsal root ganglia. The level of growth at 24 and 48 hours was calculated and normalized to growth in unmodified fibrin. Mean and standard error of the mean are shown. Legend to Figure 4: -- 2 4 Hours, -◊- 48 Hours

Figure 8 is a graph showing enhancement of neurite outgrowth with YIGSR (SEQ. I.D. NO. 3) modified fibrin gels. A concentration series of YIGSR (SEQ. I.D. NO. 3) cross-linked into fibrin was tested in triplicate with day 8 chick dorsal root ganglia. The level of growth at 24 and 48 hours was calculated and normalized to growth in unmodified fibrin. Mean and standard error of the mean are shown. Legend to Figure 5: -- 2 4 Hours, -◊- 48 Hours

Figure 9 is a graph showing that peptides grafted in combination at a total concentration of 8 mol peptide/mol fg. HAV (SEQ. I.D. NO. 6) demonstrated a maximum efficacy at 2 mol/mol fg. Therefore, HAV (SEQ. I.D. NO. 6) was incorporated at this concentration and the second peptide shown was incorporated at 6 mol/mol fg. The growth of neurites relative to growth in unmodified fibrin is shown in the first bars of this graph. The normalized growth of the second peptide grafted alone is shown as well as the theoretical additive growth derived from the two peptides grafted alone. Mean and standard error of the mean are shown. Legend to Figure 6: Combination Growth, ■ Growth Alone, ▩ Additive Results from Growth Alone.

Figure 10 is a graph showing that peptides grafted in combination at a total concentration of 8 mol peptide/mol fg. RGD (SEQ. I.D. NO. 2) demonstrated a maximum efficacy at 2 mol/mol fg. Therefore, RGD (SEQ. I.D. NO. 2) was incorporated at this concentration and the second peptide shown was incorporated at 6 mol.mol fg. The growth of neurites relative to growth in unmodified fibrin is shown in the first bars of this graph. The normalized growth of the second peptide grafted alone is shown as well as

the theoretical additive growth derived from the two peptides grafted alone. The final series of data labeled laminin represents the four relevant laminin derived peptides, RGD (SEQ. I.D. NO. 2), IKVAV (SEQ. I.D. NO. 1), RNIAEIIKDI (SEQ. I.D. NO. 5) and YIGSR (SEQ. I.D. NO. 3), these peptides were grafted in equimolar quantities at 2 mol peptide/mol fg. Mean and standard error of the mean are shown. Legend to Figure 7: G r afted with RGD, ■ Sample Grafted Alone, ▩ Cumulative Value for Sample and RGD.

Figure 11 is a graph comparing heparin binding peptides derived from three separate proteins, NCAM (SEQ. I.D. NO. 7), platelet factor 4 and antithrombin III were cross-linked into fibrin at 8 mol/mol fg. The level of neurite outgrowth at 48 hours was normalized against growth in unmodified fibrin and is shown below. Additionally, controls of crosslinked peptide with heparin or just soluble peptide were tested. The growth is shown below with levels that are significantly different from unmodified fibrin ($p<0.05$) noted with a star. Mean and standard error of the mean are shown. Legend for Figure 8: ▩ Peptide Grafted at 8mol/mol Fg, P e p tide Grafted at 8 mol/mol Fg with Soluble Heparin, ▤ Peptide Added in Soluble Phase.

Detailed Description of the Invention

Growth Factors and Peptides which bind Heparin

Low-Affinity heparin binding Growth Factors

Examples of low heparin-binding affinity growth factor include neurturin, persephin, IGF-1A, IGF-1, EGF, NGF, NT-3, BDNF, NT-4, TGF-beta2, TGF-beta3, TGF-beta4, or a peptide fragment of any one of these. Other growth factors may be found which contain similar basic domains that are not enumerated here.

"Low-heparin-binding affinity" of a growth factor or peptide fragment thereof is defined as any protein, peptide, or derivative or combination thereof, that is capable of demonstrating the biological activity of a growth factor, and that has a relatively binding low affinity for binding heparin, and will elute from a heparin-affinity column at sub-physiological NaCl concentrations. Physiological levels of NaCl may be defined as about 140 mM NaCl. Herein the term "sub-physiological" levels of NaCl, therefore, may be further defined as from between about 25 mM to about 140 mM NaCl. Although low heparin-binding affinity growth factors elute from heparin-affinity columns at sub-physiological NcC1 concentrations, their low affinity for heparin can still be used to sequester the protein or peptide to a matrix that contains heparin or a heparin-binding site. Peptides that bind heparin with high affinity have a characteristic amino acid domain that will not elute from a heparin-affinity column at less than 140 mM NaCl.

Peptides useful in promoting the controlled release of low affinity-heparin-binding growth factors include those having a low heparin-binding domain of about 8 to about 30 basic amino acids in length. In a preferred embodiment, this includes at least 2 basic amino acid residues, a ratio of basic to acidic amino acid residues of at least 2, and a ratio of hydrophobic amino acid residues to basic amino acid residue of at least 0.67. Representative peptides are listed in Table 1b, identified as SEQ. ID. NO.:1, SEQ ID. NO.: 2; SEQ ID. NO.: 3; SEQ ID. NO.: 4; and SEQ ID. NO.: 5. The standard one letter abbreviations are used, as shown in Table 1a. As generally referred to herein, basic amino acids include K (lysine) or R

(arginine); acidic amino acid residues include D (aspartic acid) or E (glutamic acid); and hydrophobic amino acid residues include A (alanine), V (valine), F (phenylalanine), P (proline), M (methionine), I (isoleucine), or L (leucine). C (cysteine) amino acids that are involved in a disulfide bridge are also considered hydrophobic.

Table 1a: Heparin binding Sequences

SEQ ID NO: 1	ATIII domain	K(ßA)FAKLAARLYRKA
SEQ ID NO: 2	PF4 domain	YKKIIKKL
SEQ ID NO: 3	NCAM domain	KHKGRDVILKKDVR
SEQ ID NO: 4	ATIII domain modified	R(ßA)FARLAARLYRRA
SEQ ID NO: 5	bFGF domain	KDPKRLYRSRKY
SEQ ID NO: 6	NGF basic domain	CVLSRKAVRRA
SEQ ID NO: 7	NT-3 basic domain	CALSRKIGRT
SEQ ID NO: 8	BDNF basic domain	CTLTIKRGR

Table1b: Amino acid three-letter and one-letter abbreviations:

Abbreviation 3 letter	Abbreviation 1 letter	Amino Acid Name
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic Acid
Cys	C	Cysteine
Glu	E	Glutamic Acid
Gln	Q	Glutamine
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

Higher Affinity heparin binding peptides and binding domains

The matrices can be modified to include chemically cross-linked peptides. These peptides preferably include a sequence that mimics the heparin-binding regions of a protein such as neural cell adhesion molecule, fibronectin, laminen, midkine, and antithrombin III. These peptides are chemically cross-linked to the matrix to enhance attachment of cells to the matrix. Representative peptides include HAV (SEQ. I.D. NO. 6) bound to the matrix at a concentration of about 2 mol peptide/mol fibrin gel; RGD (SEQ. I.D. NO. 2) bound to the matrix at a concentration of about 1.5 mol RGD/peptide mol fibrin gel; IKVAV (SEQ. I.D. NO. 1) bound in a concentration of about 8 mol peptide/mol fibrin gel; YIGSR (SEQ. I.D. NO. 3) at a concentration of about 6 mol peptide/mol fibrin gel and RNIAEIIKDI (SEQ. I.D. NO. 5) at a concentration of about 8 mol peptide/mol fibrin gel, or mixtures thereof of two or more peptides or fusion peptides including these or similar peptides. In one preferred embodiment for neurite extension, HAV (SEQ. I.D. NO. 6) and RNIAEIIKDI (SEQ. I.D. NO. 5) are included at a ratio of about 1:3. In another embodiment RGD (SEQ. I.D. NO. 2) and YIGSR (SEQ. I.D. NO. 3) or DGEA (SEQ. I.D. NO. 4) are combined with a ratio of about 1:3. In still another example, mixtures of peptides include RGD (SEQ. I.D. NO. 2), YIGSR (SEQ. I.D. NO. 3), and RNIAEIIKDI (SEQ. I.D. NO. 5) at a ratio of about 1:1:1, respectively.

In still another embodiment, the peptide includes two or more different domains, at least one of which is a heparin-binding domain and at least one additional domain is a bioactive molecule or binds to another molecule, i.e., includes a second binding domain. Examples of other binding domains K(A)FAKLAARLYRKA (SEQ. I.D. NO. 8), YKKIIKKL (SEQ. I.D. NO. 9), KHKGRDVILKKDVR (SEQ. I.D. NO. 10), or a mixture thereof. An exemplary heparin binding domain from ATIII is SEQ. I.D. NO.18. In one embodiment, the preferred heparin-binding domain elutes from a heparin-affinity column at [NaCl] greater than 0.34 mol. Others

include alpha 2 protease inhibitor and a modified aprotinin with a Factor XIIIa substrate site. These peptides may also include an enzymatic cleavage site, most preferably having a low kcat and a high km. An Exemplary chimeric peptide includes a first C-terminal domain comprising a heparin-binding peptide sequence of antithrombin III; and a second N-terminal domain comprising an alpha 2-plasmin inhibitor substrate for Factor XIIIa.

Matrix Materials

The matrix itself may also comprise any of a variety of materials, including a protein such as fibrin, collagen, hyaluronic acid, or a synthetic polymer hydrogel, such as a poly (ethylene glycol) hydrogel or a derivative or a mixture thereof, or any variety of synthetic derivatives thereof, that is capable of supporting the attachment of the types of peptides and growth factors described herein. Synthetic polymer hydrogels, including hydrogels formed by photopolymerization or conjugate addition reactions, can be utilized as the substrate for the delivery device. This synthetic material may contain cell adhesion domains, substrates for enzymatic degradation or hydrolysis, heparin-binding domains, or covalently bound heparin. Through either the covalent or non-covalent attachment of heparin or sites which have heparin-like binding, such synthetic matrices can bind low heparin-binding affinity growth factor proteins and release them in a controlled manner. Release can occur by degradation of matrix components or dissociation of the low heparin-binding affinity growth factor proteins, just as in protein matrices. The substrate for the delivery system can also include matrices of hyaluronic acid or hyaluronic acid derivatives.

Heparin or Heparin-like Polysaccharides

Preferred heparin or heparin-like polysaccharides have a molecular weight of at least 3,000 Daltons. For practical purposes a molecular weight maximum of 10,000,000 may be considered. Most preferred polyscharrides have at least one negative charge per two saccharide rings and no more than one positive charge per ten saccharide rings. Examples of heparin-like polysaccharides include dextran sulfate, chondroitin sulfate, heparin sulfate, fucan, alginate or derivatives thereof. It is most preferred that the molar ratio of heparin to growth factor or peptide, be in the range of 1, but will be higher

if the growth factors or peptides are to be retained for longer periods of time, or lower, if they are to be released faster. These heparin and heparin-like polysaccharides may be either covalently attached to or immobilized via non-covalent interactions (i.e. electrostatically bound) to the matrix or other substrate. Synthetic polymers may be designed that function in a heparin-like manner.

Other heparin-like polymers include, for example, dextran sulfate, chondroitin sulfate, heparin sulfate, fucan and alginate (See Maaroufi, et al, (1997) *Biomaterials* 18:359-366) and Logeart, et al., (1997) *Eur J Cell Biol* 74:376-384). In addition, synthetic heparin-like polymers or polysaccharide derivatives also exist, which have similar binding affinity for heparin-binding proteins or peptides as heparin. Examples of heparin-like polysaccharide derivatives include dextran derivatives such as those made by de Raucourt, et al., (1998) *J. Biomed Mater Res* 41 :49-57) and Bagheri-Yamand, (1998) *Br. J. Cancer* 78:111-118). Examples of heparin-like synthetic polymers include those by Silver, et al., (1992) Biomaterials 1992, 13:339-344). As used herein, the term "heparin" is considered to include all heparin-like polymers and polysaccharides including those described above.

Means for modifying matrix release and/or degradation properties

As demonstrated by the examples, one can control the cell-mediated rate of fibrin degradation, and thereby control release and/or degradation properties of the matrix. One method to control the degradation of the material is to modify the structural characteristics through the initial concentration of the precursor components. By making a denser matrix, the rate of cellular infiltration, and related cellular degradation, will be decreased. This can be accomplished by increasing the density of the gel through either an increase in the polymer (such as fibrinogen) concentration or an increase in the cross-linking (for example, through the amount of factor XIIIa added to the fibrinogen).

Another example involves controlling the rate of degradation by crosslinking exogenous protease inhibitors into the polymer forming the matrix. In order to maximize the efficacy of these inhibitors, they must be

covalently bound to the gel to provide a high local concentration. Proteases can be either selected or engineered to have a factor XIIIa substrate site. In the example of 2-plasmin inhibitor, a substrate sequence is already present. For other proteins, like aprotinin, this site can be built into the protein. These proteins will then be added to the coagulation mixture and allowed to cross-link into the fibrin. The inhibitors will remain in the gel through this covalent cross-link until enzymatic degradation of the fibrin begins. This initial degradation will then release the bound inhibitors, preventing widespread degradation of the fibrin matrix. This method can also be further modified to incorporate an enzymatic degradation site in the protease inhibitor between the cross-linking domain and the active protein domain. This method will then allow the protease inhibitor to be released free of fibrin degradation products, potentially increasing the efficacy of the released inhibitor.

Another example involves cross-linking bi-domain peptides into the polymer, for example, fibrin that includes a factor XIIIa substrate sequence and a protease binding domain. The binding domain in the peptide can be a degradation site that is selected to have a low Km and a low Kcat leading to high level of enzyme binding at the site but a low level of enzymatic activity. This then serves to allow the incorporated peptides to act as a competitive inhibitor for fibrin degradation sites. However, since the incorporated peptide would be cleaved at a very slow rate, it would effectively immobilize the protease and prevent it from degrading the fibrin matrix. Additionally, the binding domain could interact with the protease through a site unrelated to the enzymatic activity. (i.e. heparin binding) This would then allow the protease to be sequestered in the fibrin before it could degrade the gel as well.

Applications

The matrix having peptides and/or growth factors bound thereto can be used for tissue engineering, for repair or replacement of tissues. In one embodiment, the matrix is seeded with cells prior to or at the time of implantation at a site in need thereof. In another embodiment, the matrix is used for delivery of growth factors to enhance repair or regeneration of the

cells and/or tissues at or adjacent to the site where the matrix is implanted. The growth factor or peptide fragment thereof provided in the matrix is released by the degradation of a component of the matrix, by the disassociation of growth factor from the heparin or heparin-like polysaccharide, or by a combination of these mechanisms. This results in a sustained and controlled release of growth factor into the site where the matrix is implanted. The growth factors are selected based on the particular application. For example, TGF-beta3 is particularly useful in dermal healing.

In another embodiment, the matrix is provided on, within, or via a device such as a vascular graft or shunt, or prosthetic.

The following examples are included to demonstrate preferred embodiments of the invention.

Example 1: Matrices incorporating strong heparin binding factors.

The release system was first characterized with growth factors that demonstrate strong heparin binding affinity. The results of these studies are shown in Figure 1. Bar 1 shows the neurite extension through unmodified fibrin gels. Bar 2 shows that the release system, consisting of heparin and incorporated heparin-binding peptide, does not promote neurite extension without the addition of growth factor. Bars 3, 4 and 6 show the dose-response effect of matrix-bound bFGF, which enhances neurite extension by up to 100% (bar 6). Bars 5 and 7 show that when bFGF is added during polymerization of the fibrin gel in the absence of the release system, it does not enhance neurite extension, presumably because it diffuses out of the fibrin too quickly. Bar 8 shows that the presence of bFGF in the culture media promotes neurite extension similar to that of matrix bound bFGF at the same concentration. Bar 9 shows that VEGF does not enhance neurite extension, demonstrating that the growth factor bound must also show bioactivity in neural models to promote neurite extension. Bar 10 shows that when the amount of heparin-binding sites is decreased by 50%, the release of growth factor is not significantly affected. This deviation supports the contention that a high excess of growth factor binding sites is present. Bar 11 shows that when the peptide is cross-linked to the matrix, it will constitute

a functional heparin-based delivery system. Bar 12 demonstrates that heparin and bFGF without the immobilized heparin-binding peptide do not constitute a functional delivery system and that heparin and immobilized peptide will enhance and sustain the sustained release of growth factor. These results demonstrate that the delivery system is capable of sustained release of a heparin binding growth factor in an active form.

The ability of heparin-containing fibrin matrices to deliver low-heparin-binding growth factors has been tested using NGF-beta, NT-3 and BDNF, all members of the neurotrophin family. Fibrin gels were made as described in Example 2, except for the addition of peptide, heparin and neurotrophin described below. Fibrin gels were made containing a final concentration of 3.5 mg/ml fibrinogen, 2.5 mM Ca++, 2 NIH units/ml thrombin, 0.25 mM peptide, 0.125 mM heparin, and 0.1 μg/ml of the neurotrophin to be tested. Otherwise, the assay was performed as described in Example 2. The results are shown in Figure 2. The fibrin group represents the behavior of normal fibrin and was the control data set to which neurite extension was normalized. These ganglia were cultured in the presence of 20 ng/ml NGF. All other treatments contained no growth factors in the media. For each of the three neurotrophins tested, neurite extension was enhanced only when the heparin-based delivery system of peptide and heparin was present and bound to the matrix ("bound" bars). The addition of neurotrophin alone ("soluble" bars) without peptide or heparin did not enhance neurite extension presumably because the factor diffused out of the fibrin too quickly. The materials shown in Figure 2 differ from those in Figure 1 in that "low-heparin-binding affinity" growth factors were released in Figure 2, while the factors released in Figure 1 are considered strongly heparin-binding. This distinction is clearly demonstrated in Example 1, in which the heparin-binding growth factor (bFGF) eluted from heparin above physiological NaCl concentrations, whereas the low and non-heparin-binding growth factors (NGF- , and TGF- 2) eluted at sub-physiological NaCl concentrations. In both cases, the heparin-based delivery systems were able to deliver bioactive growth factors in a controlled manner. This

demonstrates, surprisingly, that non-heparin-binding growth factors can be released in a sustained manner from heparin-containing matrices.

To determine how long the release of low-affinity growth factors could be sustained, NGF-beta-containing gels were washed for extended time periods prior to cell seeding. In each case, the gels were washed thoroughly with TBS. The results are shown in Figure 3. The bioactivity of the growth factor present after 4 days is the same as after 1 day of washing. After 1 week of washing, the bioactivity of the growth factor released is decreased.

These results demonstrate that NGF-beta, NT-3 and BDNF are being sequestered by heparin and that the increase in outgrowth versus unmodified fibrin is due to matrix bound growth factor, rather than free growth factor. This demonstrates that such heparin-based materials can be used to sequester proteins with low heparin-binding affinity, which contain exposed basic regions. In this three-dimensional fibrin matrix, heparin was immobilized at approximately 380 μg/ml. This material demonstrated NGF release over approximately one week. There are situations when release for as little as a day would be therapeutically useful. Thus a useful amount of immobilized heparin would be at least 95 μg/ml, with higher amounts leading to release sustained over longer periods.

The results also demonstrate that "non-heparin-binding growth factors", such as NGFbeta, BDNF and NT-3, can be released in a controlled manner from heparin-based drug delivery systems based on their low affinity for heparin. These proteins can be sequestered within three-dimensional materials containing immobilized heparin based on basic domains found in the proteins. Furthermore, the growth factors released from these systems retain bioactivity in *in vitro* models as shown above. This demonstrates other low heparin-binding affinity growth factor proteins containing similar types of basic domains could be released from heparin-based delivery systems in a similar manner.

Example 2: Members of the TGF-beta family.

The approach to sequence analysis described above can be applied to growth factors from other families as well. A list of growth factors and their

sequences is shown in Table 2, which shows domains that may have low heparin-binding affinity and could be released from heparin-based delivery systems such as those described above. These factors include some members of the TGF-beta family, namely TGF-beta2, TGF-beta3, and TGF-beta4. TGF-beta2, TGF-beta3 and TGF-beta4 are members of a family, which contains one strongly heparin-binding growth factor, TGF-beta1. However, other members of the TGF-beta family have been reported in the literature to have lower heparin-binding affinity, such as TGF-beta3. TGF-beta1 has been shown to be heparin-binding at physiological ionic strength, i.e. at 140 mM NaCl. TGF-beta3 lacks a key charge and has been demonstrated to be "non-heparin-binding" at physiological conditions. Heparin-affinity chromatography on TGF-beta2 demonstrates that it also possesses low-heparin-binding affinity under physiological conditions. The basic domain in each of these growth factors, which could potentially interact with heparin, is underlined in the list of sequences given in Table 2.

Some members of a growth factor family may be heparin-binding, while others are not, as is demonstrated by the TGF-beta family. Despite the low-heparin-binding affinity of such "non-heparin-binding" growth factors, they may still be released in a controlled manner from heparin-based delivery systems if they contain a basic domain and the number of heparin-binding sites present in the system is in relatively greater excess to the amount of growth factor to be bound.

Other families of growth factors also contain growth factors which are not reported in the literature to be heparin-binding, but which contain basic domains (Shown in Table 3).

Table 3: GROWTH FACTOR SEQUENCES

TGF-beta1 ALDTNYCFSS TEKNCCVRQL YIDFRKDLGW KWIHEPKGYH ANFCLGPCPY IWSLDTQYSK VLALYNQHNP

TGF-beta2 ALDAAYCFRN VQDNCCLRPL YIDFKRDLGW KWIHEPKGYN ANFCAGACPY LWSSDTQHSR VLSLYNTINP

TGF-beta3 ALDTNYCFRN LEENCCVRPL YIDFRQDLGW KWVHEPKGYY ANFCSGPCPY LRSADTTHST VLGLYNTLNP

TGF-beta1 GASAAPCCVP QALEPLPIVY YVGRKPKVEQ LSNMIVRSCK CS

TGF-beta2 EASASPCCVS QDLEPLTILY YIGKTPKIEQ LSNMIVKSCK CS

TGF-beta3 EASASPCCVP QDLEPLTILY YVGRTPKVEQ LSNMVVKSCK CS

TGF-beta4 FSQS FREVAGRFLA SEASTHLLVF GMEQRLPPNS ELVQAVLRLF QEPVPQGALH RHGRLSPAAP KARVTVEWLV RDDGSNRTSL IDSRLVSVHE SGWKAFDVTE AVNFWQQLSR PPEPLLVQVS VQREHLGPLA SGAHKLVRFA SQGAPAGLGE PQLELHTLDL RDYGAQGDCD PEAPMTEGTR CCRQEMYIDL QGMKWAKNWV LEPPGFLAYE CVGTCQQPPE ALAFNWPFLG PRQCIASETA SLPMIVSIKE GGRTRPQVVS LPNMRVQKCS CASDGALVPR RLQHRPWCIH

G[redacted]NF SPD KQMAVLPRRE RNRQAA[redacted]ANP ENSRGKGRRG QRGKNRGCVL TAIHLNVTDL GLGYETKEEL IFRYCSGSCD

Neurturin LGA RPCGLRELEV RVSELGLGYA SDETVLFRYC AGACEAAARV YDLGLRRLRQ RRRLRRERVR AQPCCRPTAY

GDNF AAETTYDKIL KNLSRNRRLV SDKVGQACCR PIAFDDDLSF LDDNLVYHIL RKHSAKRCGC I

Neurturin EDEVSFLDAH SRYHTVHELS ARECACV

NT-4 GVSETAPASR RGELAVCDAVSG

NGF-beta SSSHPIFHRG EFSVCDSVSV WVGDKTTATD IKGKEVMVLG EVNINNSVFK QYFFETKCRDP NPVDSGCRGID

BDNF HSDPARRGEL SVCDSISEWV TAADKKTAVD MSGGTVTVLE KVPVSKGQLK QYFYETKCNP MGYTKEGCRGID

NT-3 YAEHKSHRGEY SVCDSESLWV TDKSSAIDIR GHQVTVLGE IKTGNSPVK QYFYETRCKE ARPVKNGCRGID

NT-4 WVTDRRTAVD LRGREVEVLG EVPAAGGSPL RQYFFETRCK ADNAEEGGPG AGGGGCRGVD RRHWVSECVD

NGF-beta SKHWNSYCTT THTFVKALTM DGKQAAWRF IRIDTACVCV LSRKAVRRA

BDNF KRHWNSQCRT TQSYVRALTM DSKKRIGWRF IRIDTSCVCT LTIKRGR

NT-3 DKHWNSQCKT SQTYVRALTS ENNKLVGWRW IRIDTSCVCA LSRKIGRT

NT-4 RRHWVSECKA KQSYVRALTA DAQGRVGWRW IRIDTACVCTL LSRTGRA

IGF-1A GP ETLCGAELVD ALQFVCGDRG FYFNKPTGYG SSSRRAPQTG IVDECCFRSC DLRRLEMYCA PLKPAKSA

IGF-1B GP ETLCGAELVD ALQFVCGDRG FYFNKPTGYG SSSRRAPQTG IVDECCFRSC DLRRLEMYCA PLKPAKSA

E[redacted] NSDSECPLSH DGYCLHDGVC[redacted]YIEALDKYA
CNCVVGYIGE RCQYRDLKWW ELR

5

Basic domains of low heparin-binding affinity growth factor proteins are underlined and basic amino acid residues (K or R) are shown in bold

Analysis of the primary protein of growth factors, such as those shown in Table 3 can be used to identity basic domains.

By analyzing the sequences of the TGFbeta family and the neurotrophin family, one skilled in the art can observe a pattern in the basic domains underlined in Table 3. From this pattern observed in the basic domains of low heparin-binding affinity proteins, an approximate formula was developed to identify similar basic domains in other proteins. The formula defines a basic domain to be of length about 8 to 30 amino acid residues, comprising at least 2 basic amino acid residues, with a ratio of basic to acidic amino acid residues to at least 2, and a ratio of hydrophobic amino acid residues of at least 0.67. Secondary and tertiary protein structure also influences the heparin-binding affinity of a basic domain within a protein or peptide, and for this reason the formula is approximate. Therefore, it is necessary to perform heparin-affinity chromatography or some other experimental technique to determine the relative heparin affinity of a protein or peptide. The term "low heparin-binding affinity protein" refers only to those proteins or peptides which elute from heparin-affinity chromatography at a NaCl concentration of less than about 140 mM. The formula for sequence analysis described above was applied to the list of growth factors found in Table 3 and used to identify basic domains which could potentially bind to heparin or heparin-like polymers.

Low heparin-binding affinity protein should dissociate rapidly from heparin under physiological conditions. However, the results shown in Figure 3 demonstrate that such low heparin-binding affinity growth factor proteins can be released in a controlled manner from heparin-based delivery systems. This result suggests that other growth factors containing domains which meet the requirements of the formula described above may also be released in a controlled manner from heparin-based delivery systems. Examples of such growth factors are shown in the sequence list in Table 3, and the basic domains of these growth factors are underlined.

Two members of the GDNF family, namely neurturin and persephin, both contain basic domains. GDNF is reported in the literature to be heparin-binding, but no reports have been made to date on the heparin-affinity of other members of the GDNF family. Neurturin and persephin,

based on the analysis presented above, would appear to have sufficient heparin binding affinity to be releasable by the methods and materials described herein.

IGF-1A and IGF-1B are members of the insulin-like growth factor family. Although there are extensive reports of insulin-like growth factor binding proteins binding to heparin, there is no documentation in the literature of IGF-1A or IGF-1B binding to heparin. Both of these proteins contain basic domains shown in Table 3. Based on the analysis presented above, would appear to have sufficient heparin binding affinity to be releasable by the methods and materials described herein.

EGF is another growth factor, which contains a basic domain, shown in Table 3, but which is not reported in the literature to be heparin-binding. In fact, the existence of a related growth factor specifically referred to as heparin-binding EGF-like growth factor suggests that EGF does not possess high heparin-affinity. The literature suggests EGF is not heparin-binding based on sequence analysis. Based on the analysis presented above, it appears to the present inventors to have sufficient heparin binding affinity to be releasable by the methods and materials described herein.

Despite the basic domains found in each of these proteins, their heparin-binding affinity is still weak and characterized by elution from heparin-affinity columns at sub-physiological NaCl concentrations (i.e. growth factors which elute between about 25 mM and 140 mM NaCl). For example, heparin-affinity chromatography was performed for NGF-beta and TGF-beta2. In both cases the proteins were found to elute at 50 mM NaCl at pH 7.4 after several column volumes of buffer, which is well below the physiological NaCl concentration. However, *in vitro* studies demonstrate that heparin-based release systems can still be used to release NGF-beta in a controlled manner, in spite of its relatively low heparin-binding affinity.

All of the growth factors described in the example contain basic domains, but lack any literature reports of heparin-binding affinity. However, based on comparison of the sequences and the results demonstrated with other non-heparin-binding growth factors of the

neurotrophin family, sustained release from heparin-based systems should be possible with virtually any growth factor having a basic domain having the characteristics of basic domains described above.

Example 3: Heparin-affinity chromatography of heparin-binding and low affinity heparin-binding growth factors.

Heparin-affinity chromatography is a method commonly used to determine the relative affinity of heparin-binding proteins. If a protein elutes at NaCl concentrations near or below physiological level (approximately 140 mM) it is not to be considered "heparin-binding" for purposes of the description of the present invention. This is because the growth factors would dissociate rapidly from heparin *in vivo*.

The relative affinity for heparin of proteins was determined by heparin-affinity chromatography, using a TSK-GEL Heparin-5PW (7.5 cm x 7.5 mm ID) column (TosoHass, Stuttgart, Germany). Samples of the protein were injected in 20 mM Tris, pH 7.4, 0.05 M NaCl. Elution was accomplished by running a gradient of NaCl from 0.05 M to 2.0 M over 30 min, and the NaCl concentration at which elution was observed was taken as a measure of the heparin-binding affinity of the protein. The relative heparin-binding affinity of proteins not previously reported in the literature to be heparin-binding was determined and compared with proteins such as bFGF and antithrombin III, which are know to be strongly heparin-binding. The results are shown in Table 4.

The two "non-heparin-binding" growth factors, TGF-beta and NGFbeta both eluted at sub-physiological NaCl concentrations, suggesting that they have relatively low heparin-binding affinity, and will rapidly dissociate from heparin under physiological conditions. The two heparin-binding proteins, antithrombin III and bFGF, elute at NaCl concentrations that are much greater than physiological levels.

Others have also used heparin to increase the activity of and prevent the degradation of growth factors. Schroeder, et al. (1997) *Affinity bound collagen matrices for the delivery of biologically active agents*, Collagen

Corporation: USA.; Schense and Hubbell (1999) *Bioconjug Chem* 10: 75-81; Lyon, et al., (1997) *J Biol Chem* 272: 1800-18006; and Lin, eta l., (1994) *J Neurochem* 63: 758-768) used heparin to increase the stability of TGFbeta and prevent loss of activity. They also attached heparin to collagen and employed the heparin-TGFbeta complex in collagen gels to show that such heparin-based systems can improve growth factor activity in vivo by controlled release (Schroeder-Tefft, et al., (1997) *J Controlled Release* 49: 291-298). TGFbeta is known to possess strong heparin binding affinity. However, such heparin-based release systems have not been tested previously with growth factors, which are considered to have low heparin-binding affinity (those which are characterized by elution from heparin-affinity columns at sub-physiological NaCl concentrations).

Analysis of the primary sequence of a variety of proteins, including growth factors, reveals that the primary sequence may contain regions that are basic in nature and that the basic residues are commonly flanked by hydrophobic residues. These sequences are generally of a similar nature to the XBBXBX heparin-binding consensus (where X is a hydropathic residue and B is a basic residue), described by Cardin and Weintraub (1989) *Atherosclerosis* 9: 21-32. However, the exact sequence of the basic regions vary from protein to protein. The three-dimensional structure for many proteins is also available, which allows the location of basic regions to be determined. In order for basic regions to be useful for sequestration of a protein, they must be located on the surface of the protein. Frequently, such regions are observed to occur in the amino or carboxy terminus of the protein.

Table 4: NaCl concentration required to elute proteins from a heparin-affinity column.

Protein	[NaCl] require to elute peptide
bFGF	2.0 M
antithrombin III	1.58 M
TGF-	0.05 M
NGF	0.05 M

Example 4: *In Vitro* Model for Bioactivity-Neurite Extension.

The present example is provided to describe the model for assaying *in vitro* bioactivity of materials described herein.

Three-dimensional fibrin gel in vitro model for assay neurite extension. Fibrinogen was dissolved in water and dialyzed into Tris-buffered saline (TBS) at pH 7.4 for 24 hr. The concentration of fibrinogen was determined by measuring the absorbance at 280 nm. Fibrin gels were made containing a final concentration of 3.5 mg/ml fibrinogen, 2.5 mM Ca^{++}, and 2 NIH units/ml thrombin in TBS. The polymerization mixture was incubated for 60 min at 37 °C, 95 % relative humidity, and 5% CO_2.

After polymerization, 1 ml TBS was added to each well. The gels were washed 5 times over 24 hr, with the first 4 wash solutions consisting of TBS and the last solution consisting of modified neural basal medium. Dorsal root ganglia (DRGs) were dissected from day 8 chick embryos and placed in Hanks-buffered salt solution. The ganglia were placed inside the fibrin gels (one per well) and the gels were incubated for 60 min at 37 °C, 95 % relative humidity, and 5% CO_2. After 60 min, 1 ml of modified neural basal medium was added to each well. The media was changed at 24 hr. Bright field images of the ganglia were taken at 24 and 48 hr. The images were analyzed to determine the average length of neurite extension, which was calculated to be the area of an annulus between the DRG body and the outer halo of extending neurites, as shown by Herbert, et al.(1996) *J Comp Neurol* 365: 380-391. Neurite extension for each experiment was normalized by the average neurite extension through unmodified fibrin gels

from the same experiment and time point. Results are shown in Figure 1, Bar 1. This study demonstrates the utility of the invention as a cell support and growth material that will enhance neurite extension in three-dimensions. This is also demonstrated of the utility the invention would have for promoting cell growth and neurite extension under conditions found *in vivo*.

Example 5: Release of bioactive neurotrophins from heparin-based delivery system.

An xxample of a growth factor that is not considered to be a heparin-binding growth factor, but that contains a basic sequence, is nerve growth factor beta (NGF-beta) growth factor. This growth factor is considered to be non-heparin-binding and has even been used as a negative control for a heparin-binding protein in heparin-binding analysis (See Lee and Lander (1991) *Proc Natl Acad Sci U S A* 88: 2768-2772. However, NGF contains a basic domain at its C-terminus amino acid position (230-241 of human NGF beta) consisting of the following residues: CVLSRKAVRRA (SEQ ID NO:6). Similar basic domains can be found in the carboxy termini of neurotrophin-3 (NT-3, CALSRKIGRT, 248-257 of human NT-3, SEQ ID NO: 7) and brain-derived neurotrophic factor (CTLTIKRGR, 238-247 of human BDNF, SEQ ID NO: 8).

The present example demonstrates that NGF beta, NT-3 and BDNF can be delivered in a sustained manner from heparin-containing fibrin matrices that contain non-covalently immobilized heparin. This system consists of covalently immobilized heparin-binding peptides cross-linked to the fibrin matrix by factor XIIIa (See Schense and Hubbell (1999) 24.

Lee, M. and Lander, A. (1991) *Proc Natl Acad Sci U S A* 88: 2768-2772) and heparin, which is non-covalently attached to these heparin-binding peptides. These materials have been shown to effectively deliver heparin-binding growth factors, such as basic fibroblast growth factor. This enzyme covalently attaches the substrate domain of the bi-domain peptide to the fibrin network during coagulation, thereby also immobilizing the heparin-binding domain of the bi-domain peptide to the fibrin network. Heparin is

also included into the coagulation mixture, and the heparin is thereby immobilized to the fibrin network by binding to the immobilized peptide.

The active domain of many proteins may in some cases be mimicked at least in part through the use of short peptide sequences derived from the active site of the protein (Massia and Hubbell (1991) *Journal of Cell Biology* 114:1089-1100, Yamada, (1991) *J. Biol. Chem.* 266:12809-12812). Through this method, the activity of a specific protein can be conferred to an otherwise nonactive surface or matrix. This method allows a much higher concentration of active sequences to be immobilized onto a surface than is found naturally. While many peptides have been shown to have a monotonic correlation between density and cellular activity, other peptides are known to reach a maximum activity at a moderate level of peptide density. The best example is migration of cells on a surface coated with RGD (SEQ. I.D. NO. 2). If the concentration of RGD (SEQ. I.D. NO. 2) is too high, the surface binds too strongly to the cells, inhibiting cellular migration. However, if the RGD (SEQ. I.D. NO. 2) density is too low, then there is not enough traction for these cells to effectively migrate across the surface, leading to a maximal migration rate at a moderate surface concentration of peptide. (DiMilla, et al., (1991) *Biophysics Journal* 60:15-37)

Many heparin binding domains have been identified (Table 5). Additionally, heparin binding regions of several proteins such as neural cell adhesion molecule, fibronectin, laminin, midkine, and anti-thrombin III have been reported to promote neurite extension on two-dimensional surfaces. (Edgar, et al., (1984) *EMBO* 3:1463-1468; Borrajo A, et al, (1997) *Bioorganic and Medicinal Chemistry Letters* 7:1185-1190; Kallapur, S., et al, (1992). *J. Neuroscience Res.* 33:538-548; Kaneda N., et al, (1996). *J. Biochem.* 119:1150-1156; Rogers S., et al, (1985) *J. Neurosci.* 5:369-378; *Bioorganic and Medicinal Chemistry Letters* 7:1185-1190) These heparin-binding domains have been reported by indirect evidence to interact with cell-surface proteoglycans by a number of methods including inhibition by soluble heparin, enzymatic removal of cell surface proteoglycans, and

biochemical inhibition of proteoglycan synthesis (Kallapur and Akeson, 1992). These peptides have only been studied in 2-dimensional systems.

TABLE 5: Heparin binding sequences

SEQ. I.D. NO.	SEQUENCE
1	IKVAV
2	RGD
3	YIGSR
4	DGEA
5	RNIAEIIKDI
6	HAV
7	NCAM
8	K(∃A)FAKLAARLYRKA
9	YKKIIKKL
10	KHKGRDVILKKDVR
11	YEKPGSPPREVVPRPRPCV
12	KNNQKSEPLIGRKKT
13	KDPKRL
14	YRSRKY
15	YKKPKL
16	AKRSSKM
17	CRKRCN
18	*LNQEQVSP* K(∃A)FAKLAARLYRKA
19	*LNQEQVSP* YKKIIKKL
20	*LNQEQVSP* KHKGRDVILKKDVR

Table 6: List of heparin binding peptides and the proteins they are derived from.

Protein	Heparin-binding domain	Reference
Anti-thrombin III	K(∃A)FAKLAARLYRKA (SEQ. I.D. NO. 8)	Tyler-Cross et al., 1994
Platelet Factor 4	YKKIIKKL (SEQ. I.D. NO. 9)	Zeuker and Katz, 1991
Neural Cell Adhesion Molecule	KHKGRDVILKKDVR (SEQ. I.D. NO. 10)	Kallapur, 1992
Fibronectin	YEKPGSPPREVVPRPRPCV (SEQ. I.D. NO. 11) KNNQKSEPLIGRKKT (SEQ. I.D. NO. 12)	Haugen, et al, 1992
bFGF	KDPKRL (SEQ. I.D. NO. 13) YRSRKY (SEQ. I.D. NO. 14)	SwissProt: P09038
aFGF	YKKPKL (SEQ. I.D. NO. 15)	SwissPROT: P05230
LPL	AKRSSKM (SEQ. I.D. NO. 16) CRKRCN (SEQ. I.D. NO. 17)	Hata, et al., 1993

Example 6: Effect of peptide-bound matrix on neurite outgrowth.

Specific selected peptide sequences were cross-linked into a three dimensional fibrin matrix with a day 8 dorsal root ganglia embedded into the gels. The neurites were grown for 48 hr, and the migration rate of the neurites extending from the ganglia was quantified for each condition at both 24 and 48 hr. This growth was then normalized to the growth in unmodified fibrin. The ability for some of these peptides to enhance neurite outgrowth was found to increase with peptide concentration, while other peptides reach a maximal enhancement at a moderate peptide concentration. Two peptides that were tested, one from N-Cadherin, the tripeptide HAV (SEQ. I.D. NO. 6) (Figure 4), and one which is present in many extracellular matrix proteins, namely RGD (SEQ ID NO 5) (Figure 8), were shown to reach a maximal effect at a moderate concentration of incorporated peptide. HAV (SEQ. I.D.

NO. 6) achieved a maximal effect at 2 mol/mol fibrin gel ("fg") while RGD (SEQ. I.D. NO. 2) achieved a maximal effect at 1.5 mol/mol fg of incorporated peptide. In contrast, the peptide sequences IKVAV (SEQ. I.D. NO. 1) (Figure 6), RNIAEIIKDI (SEQ. I.D. NO. 5) (Figure 7) and YIGSR (SEQ. I.D. NO. 3) (Figure 8) were shown to have a linear correlation between peptide concentration and the level of enhancement. YIGSR (SEQ. I.D. NO. 3), IKVAV (SEQ. I.D. NO. 1) and RNIAEIIKDI (SEQ. I.D. NO. 5) showed maximal enhancement at 6, 8 and 8 mol peptide/mol fibrinogen respectively.

Example 7: Fibrin gels with Multiple peptides.

Since two of the peptides that were tested in Example 6 were found to have maximal effect on the neuronal cell model employed at low concentrations, it is possible to incorporate these peptides at a low concentration and still observe a large neuronal effect, leaving many cross-linking sites open. The remaining sites can then be occupied with a different peptide which has it's maximal effect at a high concentration.

This was demonstrated using several peptides. In one example, HAV (SEQ. I.D. NO. 6) was cross-linked at 2 mol/mol fibrinogen in combination with the following peptides at 6 mol/mol fibrinogen: IKVAV (SEQ. I.D. NO. 1), RNIAEIIKDI (SEQ. I.D. NO. 5), YIGSR (SEQ. I.D. NO. 3) and DGEA (SEQ. I.D. NO. 4). The growth obtained with the peptides grafted together, with the peptide grafted alone and the theoretical sum derived from the results of the two peptides grafted separately is shown in Figure 9. The cross-linking of HAV (SEQ. I.D. NO. 6) with IKVAV (SEQ. I.D. NO. 1) results in a negative interaction, where the effect on neuronal outgrowth is lower than when IKVAV (SEQ. I.D. NO. 1) is grafted alone. The cross-linking of HAV (SEQ. I.D. NO. 6) with DGEA (SEQ. I.D. NO. 4) had relatively little to no additional effect on neurite extension, resulting in growth similar to when DGEA (SEQ. I.D. NO. 4) is cross-linked into the fibrin alone. The cross-linking of HAV (SEQ. I.D. NO. 6) with YIGSR (SEQ. I.D. NO. 3) had an additive effect on neurite extension.

In another example, RGD (SEQ. I.D. NO. 2) was cross-linked at 2 mol/mol fibrinogen with the same series of peptides at 6 mol/mol fibrinogen. In several examples, the effect was similar. The incorporation of RGD (SEQ. I.D. NO. 2) with IKVAV (SEQ. I.D. NO. 1) seemed to have a negative effect as the level of neurite outgrowth was similar to that in fibrin. When RGD (SEQ. I.D. NO. 2) was cross-linked with RNIAEIIKDI (SEQ. I.D. NO. 5), it had a neutral effect in that neurite growth was similar to that seen in gels modified with RNIAEIIKDI (SEQ. I.D. NO. 5) alone. Once again, grafting with YIGSR (SEQ. I.D. NO. 3) appeared to lead to an additive effect on neurite outgrowth.

One study was done where the four peptides derived from laminin were cross-linked into the fibrin at equimolar concentrations. Since 8 mol peptide/mol fibrinogen can be obtained, this material then had 2 mol/mol fibrinogen of IKVAV (SEQ. I.D. NO. 1), RGD (SEQ. I.D. NO. 2), YIGSR (SEQ. I.D. NO. 3), and RNIAEIIKDI (SEQ. I.D. NO. 5). When neurites were grown in this material, the effect led to 75% improvement, which was higher than the combined effects from the peptides grafting alone. These peptides were thus demonstrated in the present studies to act synergistically when co-grafted into fibrin. (Figure 10).

Bi-domain peptides containing both a factor XIIIa substrate and a heparin-binding domain (Table 6) were synthesized and cross-linked into fibrin gels as previously demonstrated with one variation.

Table 7: Exact sequences of bi-domain peptides containing the factor XIIIa substrate sequence and the heparin binding domain sequence.

The source for each heparin binding domain is shown.

Peptide Name	Sequence	Source
ATIII	*LNQEQVSP* K(∃A)FAKLAARLYRKA (SEQ. I.D. No. 18)	Antithrombin III
NCAM	*LNQEQVSP* YKKIIKKL (SEQ. I.D. No. 19)	neural cell adhesion molecule
PF4	*LNQEQVSP* KHKGRDVILKKDVR (SEQ. I.D. No. 20)	platelet factor 4

(italics denote factor XIIIa substrate.)

In one example, the peptide was cross-linked into the gel alone and in the second example, it was incorporated in the presence of heparin. These two methods led to gels where an identical concentration of bi-domain peptide was covalently bound to the fibrin, but the first example resulted in free heparin binding domains being present in the gel, while the addition of heparin in the example condition resulted in this domain being occupied with a heparin fragment. Therefore, the effects of interaction between the growing neurites and either a heparin binding domain or heparin itself could be determined. When the peptide was incorporated without heparin present, it was able to enhance the extension of neurites from day 8 chick dorsal root ganglia. Three bi-domain peptides, each with a different heparin binding domain, were tested and gave statistically better growth than unmodified fibrin (Figure 11). The level of improvement ranged from 75% to 25%. This level of enhancement could be correlated to the binding affinity of the heparin binding domain that was incorporated (Table 8). When heparin was added at the beginning of the study, it was discovered that this abolished the effect, resulting in growth similar to that seen in unmodified fibrin.

Table 8: Results of the relative heparin binding affinity and

enhancement of three dimensional neurite outgrowth for several heparin binding peptides.

There is a correlation between the binding strength for each peptide and the percent enhancement of neurite outgrowth.

Peptide Name	Elution from Heparin Affinity Column (Mol NaCl)	Enhancement of Neurite Outgrowth (%)
ATIII	0.67	73.1
NCAM	0.35	24.5
PF4	0.34	20.2

Table 9: Tissue Binding Sequences

SEQ. I.D. NO.	SEQUENCE
1	IKVAV
2	RGD
3	YIGSR
4	DGEA
5	RNIAEIIKDI
6	HAV
7	NCAM
8	K(∃A)FAKLAARLYRKA
9	YKKIIKKL
10	KHKGRDVILKKDVR
11	YEKPGSPPREVVPRPRPCV
12	KNNQKSEPLIGRKKT
13	KDPKRL
14	YRSRKY
15	YKKPKL
16	AKRSSKM
17	CRKRCN
18	*LNQEQVSP* K(∃A)FAKLAARLYRKA
19	*LNQEQVSP* YKKIIKKL
20	*LNQEQVSP* KHKGRDVILKKDVR

Example 8: Custom-Designed gel matrix for neurite growth.

There are four components necessary for creating a cross-linked fibrin gel; fibrinogen, calcium, thrombin and factor XIIIa, and the structural characteristics of the material. These four components can be modified by changing the concentration of any one of them. There are two main characteristics that determine the structure of the fibrin; the density of the fibrin bundles and the thickness of each individual bundle. These two properties will then control the ability of cells to infiltrate the matrix.

Increasing fibrin concentration from 5-15 mg/mL in the precursor mixture was found to result in fibrin gels with smaller fibrin bundles that are much denser. This resulting material has been shown to be more difficult for neurites to migrate through. When the calcium concentration was increased from 2-10 mM, the fibrin bundles got thicker, but the spacing between these bundles became greater. Changing the fibrin density clearly can have a direct effect on cellular migration while changes in the fibril morphology does not. Degradation of the fibrin matrix is dependent on the morphology of these fibers. Therefore, the ability for cells to infiltrate the fibrin and the overall degradation of the gel can be controlled independently. The kinetics of fibrin formation are dependent on the amount of the two enzymes, thrombin and factor XIII, that are present. Increasing the concentration of thrombin decreases time for gelatin while increasing the factor XIII concentration increases the rate of cross-link formation. By varying the concentrations of these four precursor components, the fibrin morphology and kinetics are manipulated to provide a matrix with optimal properties.

What is claimed is:

1. A method for controlled release of molecules comprising providing a matrix binding the molecules to be released, the molecules binding directly or indirectly to the matrix via heparin binding sites, wherein the rate of release is controlled by the ratio of molecules to heparin binding domains, the number of heparin domains, and the amount of heparin available for binding.

2. The method of claim 1 wherein the molecules to be released are growth factors.

3. The method of claim 1 wherein the matrix is formed of polymer having incorporated therein or bound thereto heparin binding domains.

4. The method of claim 3 wherein the matrix is formed of a polymer having bound thereto heparin or a heparin-like polymer or fragments thereof including heparin-like binding domains.

5. The method of claim 1 wherein the molecules are bound to the matrix via heparin-binding peptides.

6. The method of claim 1 wherein the heparin-binding peptides further comprising binding sites for the molecules to be released or are covalently bound to the molecules to be released.

7. A matrix for use in the method of claim 1-6.

8. The matrix of claim 7 for promoting nerve growth.

9. The matrix of claim 7 comprising a fibrin gel.

10. The matrix of claim 9 comprising growth factors.

11. The matrix of claim 9 comprising peptides.

12. The matrix of claim 9 wherein the peptides comprise domains, wherein the domain of the growth factor or peptide fragment thereof is further defined as comprising a length of about 8 to 30 amino acid residues comprising at least 2 basic amino acid residues, a ratio of basic to acidic amino acid residues of at least 2, and a ratio of hydrophobic amino acid residues to basic amino acid residues of at least 0.67.

13. The matrix of claim 7 wherein the growth factor or peptide fragment thereof is neurturin, persephin, IGF-1A, IGF-1beta, EGF, NGFbeta, NT-3, BDNF, NT-4, TGF-beta2, TGF-beta3, or TGF-beta4.

14. The matrix of claim 7 comprising at least one peptide selected from the group consisting of HAV, IKVAV, RNIAEIIKDI, YIGSR, DGEA, a combination thereof, a combination of peptide fragments comprising HAV, IKVAV, RNIAEIIKDI, YIGSR, and DGEA, or a fusion peptide comprising said peptides.

15. The matrix of claim 7 comprising a bi-domain peptide comprising a first domain of a heparin-binding domain and a second domain consisting essentially of a Factor XIIIa substrate or a bioactive peptide.

FIGURE 1

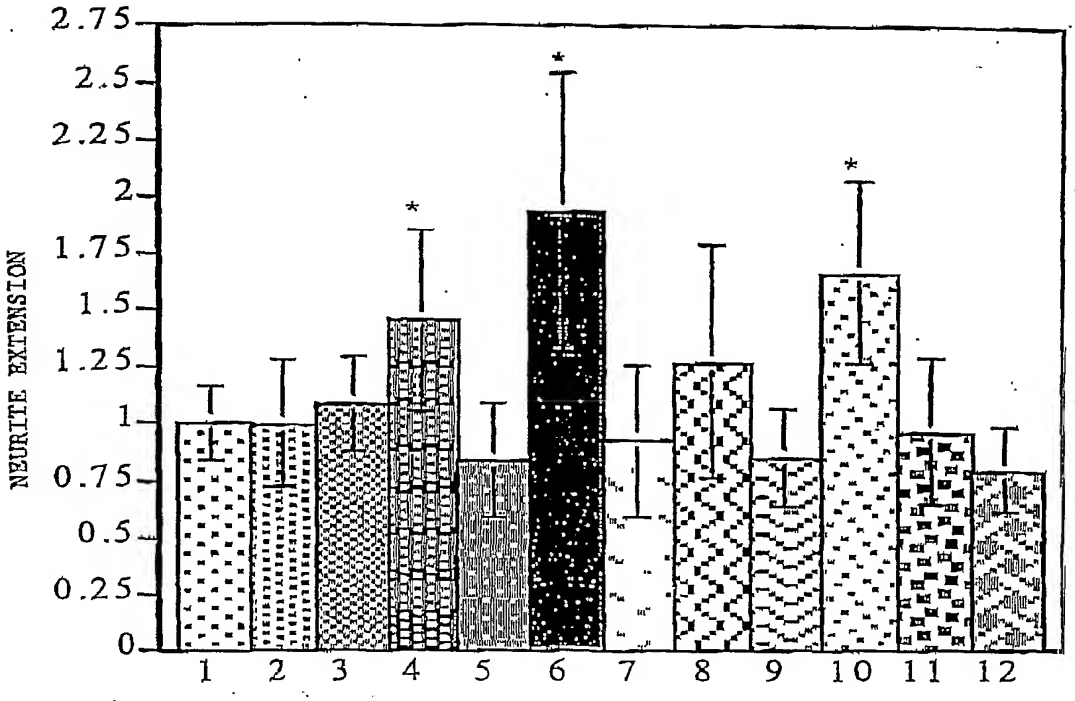


FIGURE 2

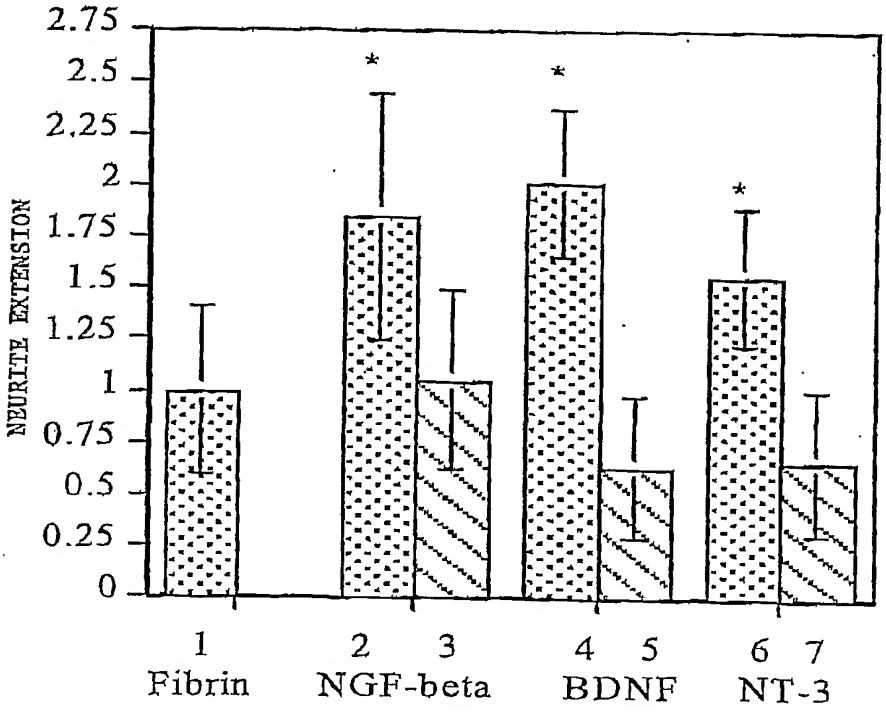


FIGURE 3

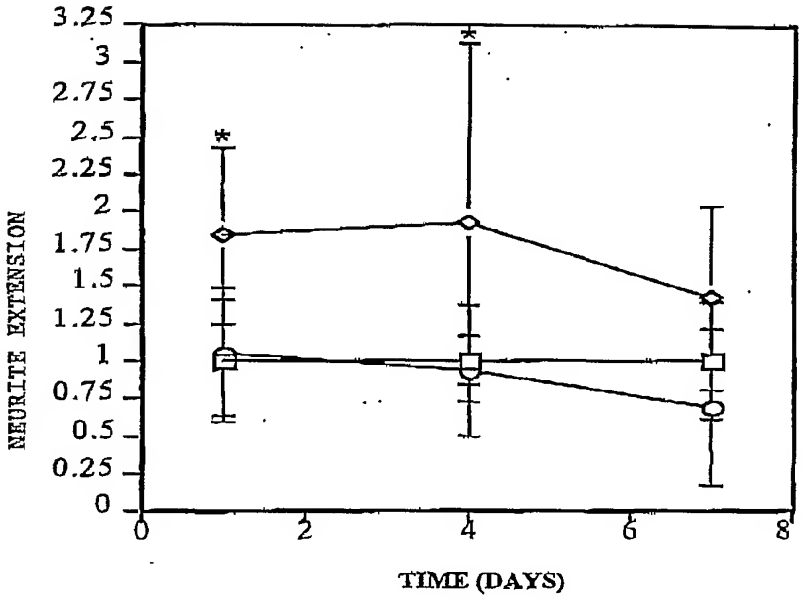


FIGURE 4

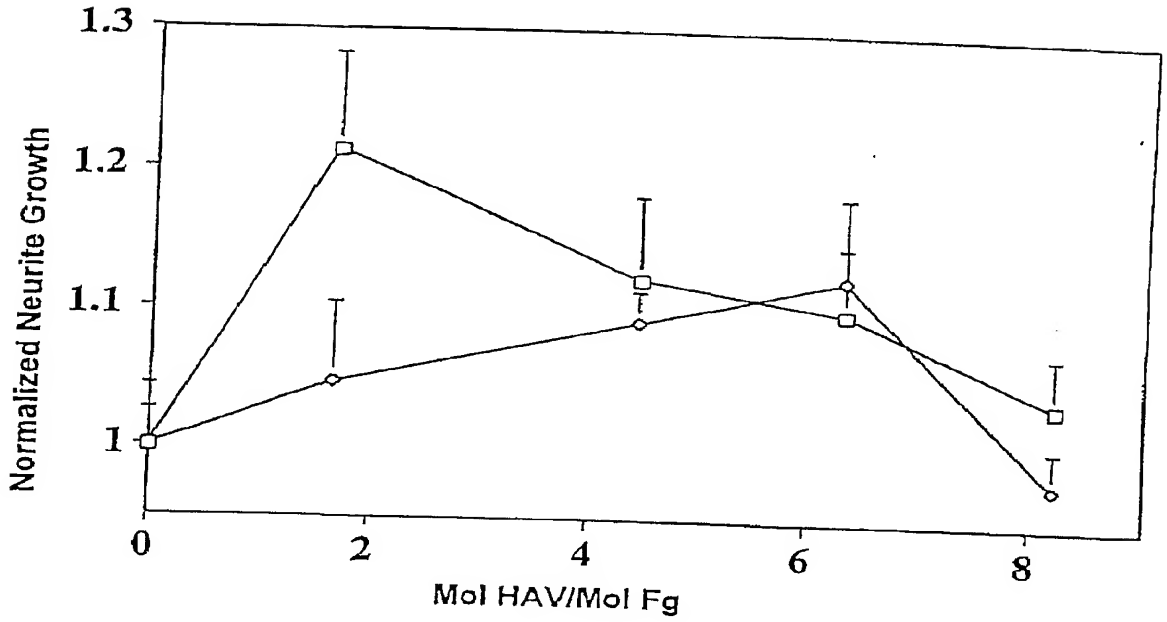


FIGURE 5

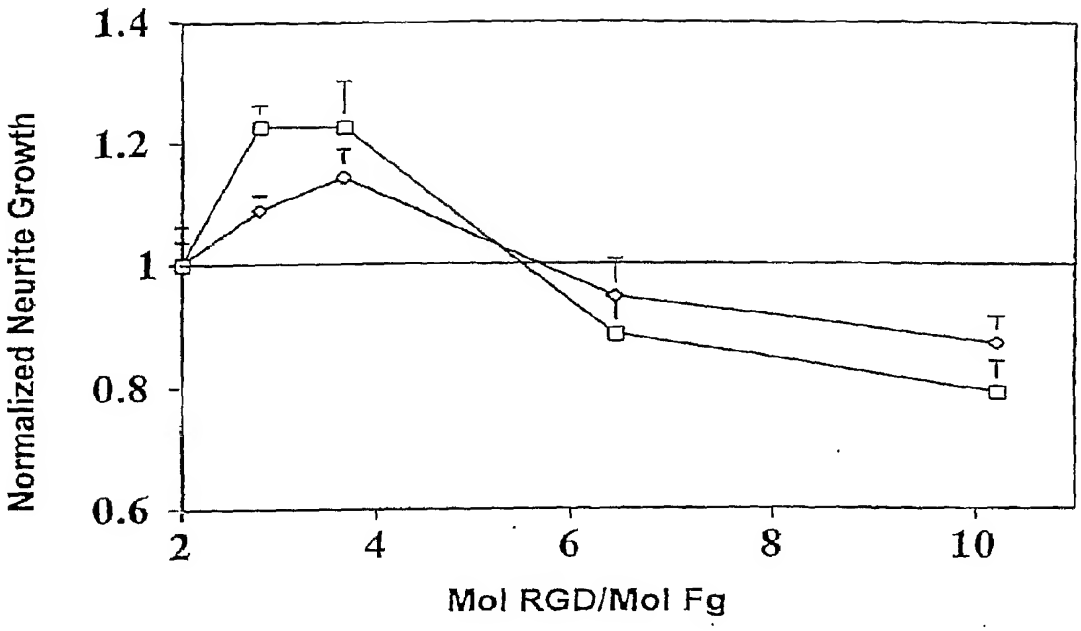


FIGURE 6

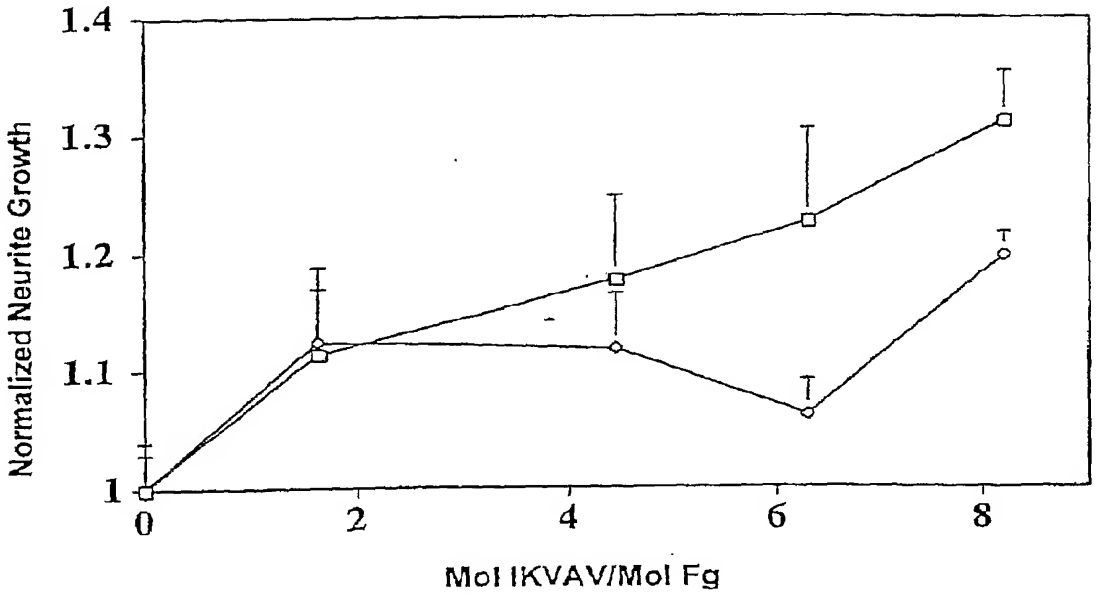


FIGURE 7

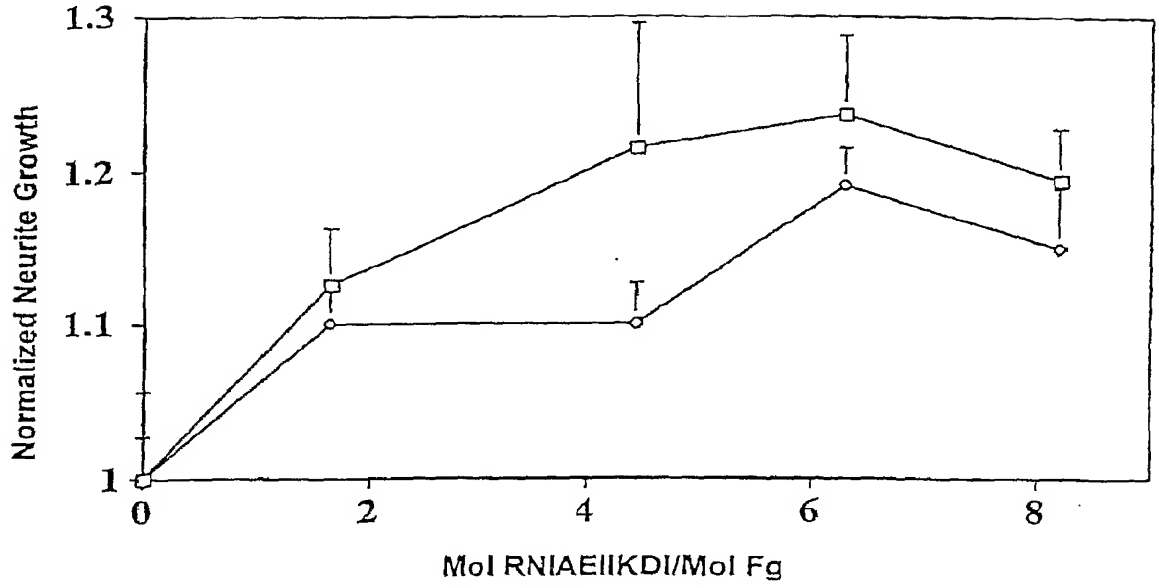


FIGURE 8

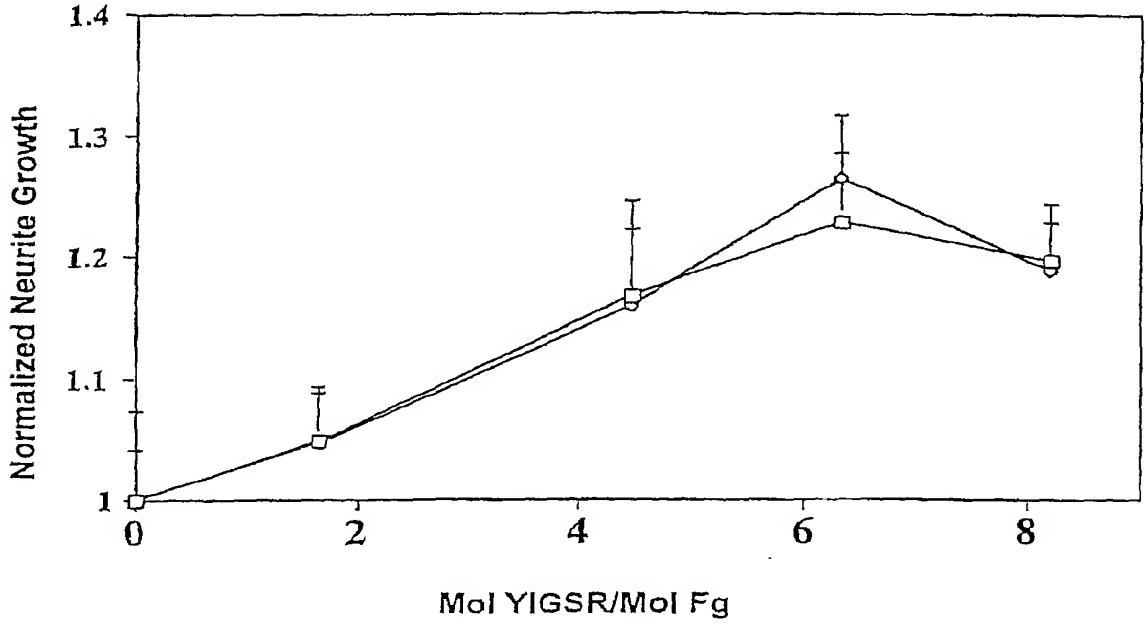


FIGURE 9

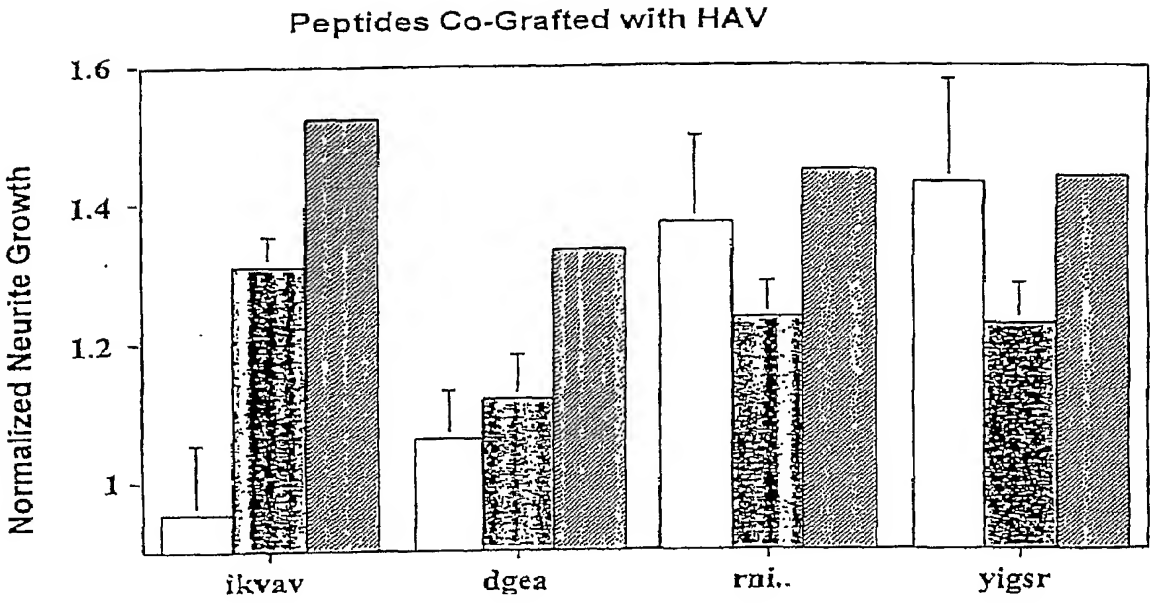


FIGURE 10

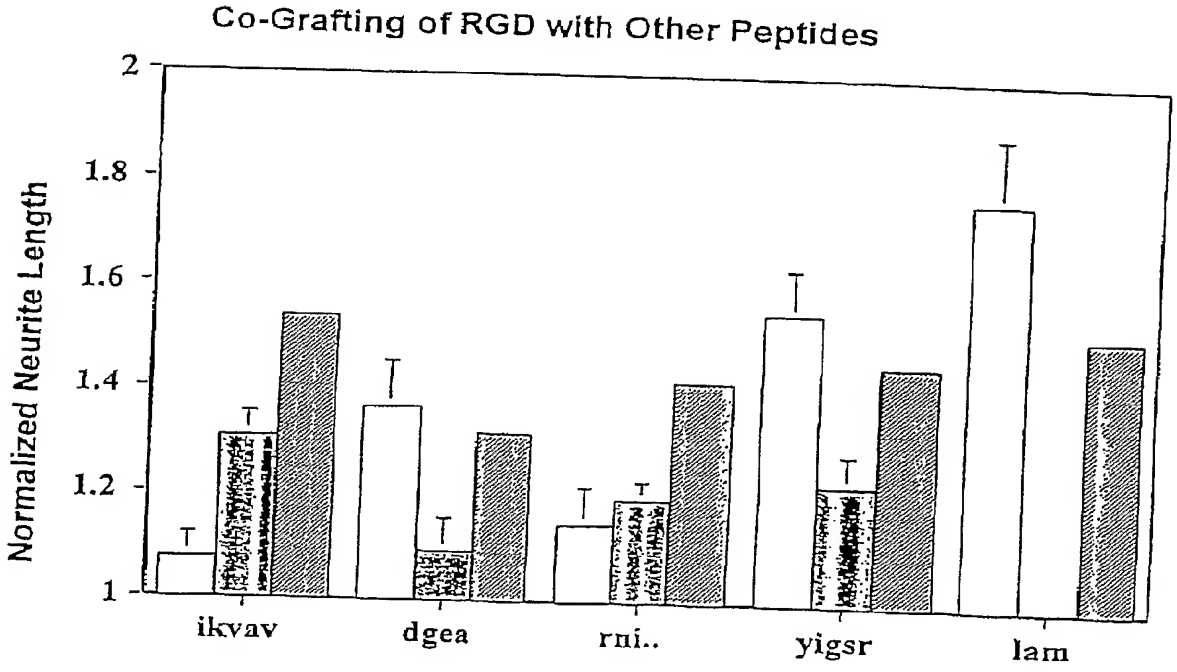
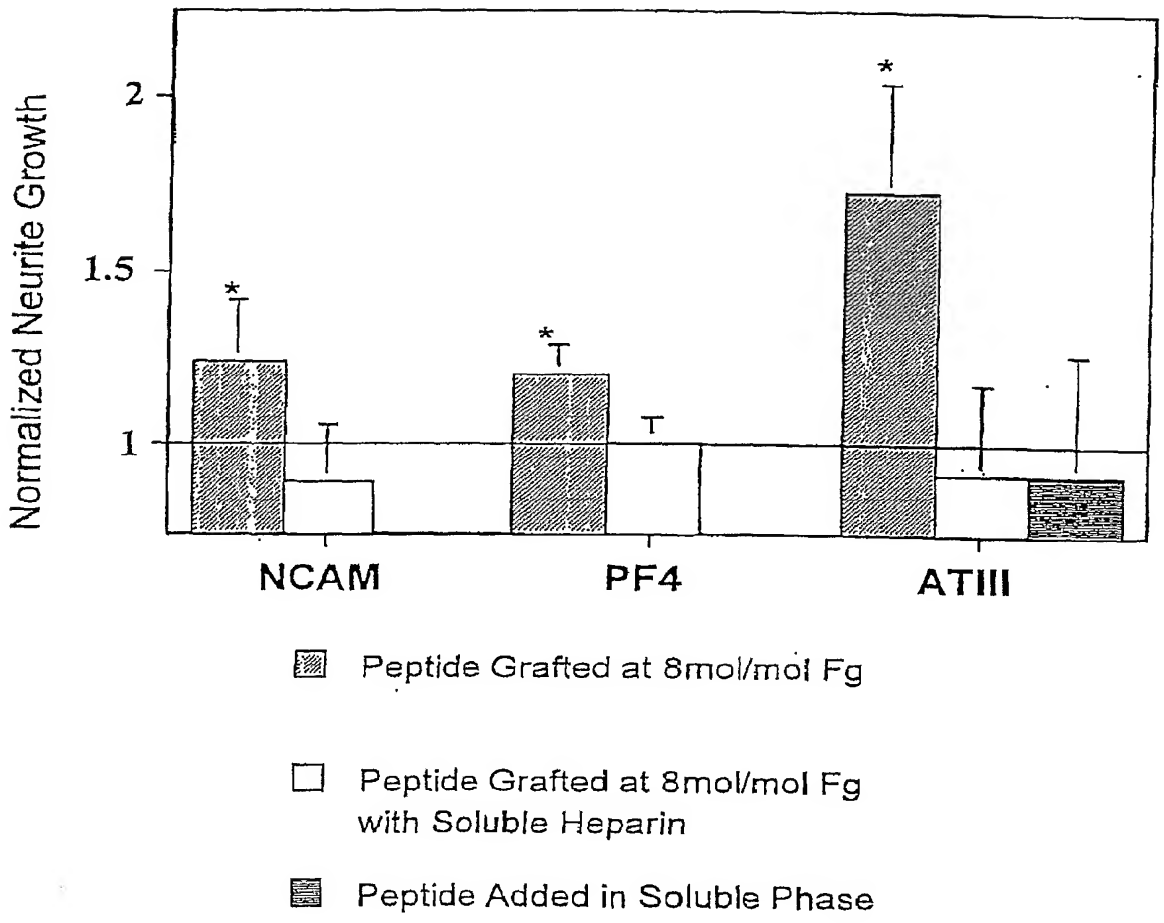


FIGURE 11


Normalized Neurite Growth
2
1.5
1
*
*
*
NCAM
PF4
ATIII
Peptide Grafted at 8mol/mol Fg
Peptide Grafted at 8mol/mol Fg with Soluble Heparin
Peptide Added in Soluble Phase

INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 00/11044

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61L27/54 A61L27/20 A61L27/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61L C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, COMPENDEX

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X	WO 92 09301 A (GREISLER HOWARD P ;AMERICAN NAT RED CROSS (US)) 11 June 1992 (1992-06-11) claims	1-11,13

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
28 July 2000	04/08/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Thornton, S

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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